Methods of Nitric Oxide detection in plants: A commentary.

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Abstract:

Over the last decade nitric oxide (NO) has been shown to influence a range of processes in plants. However a basic requirement of the scientific approaches; the ability to measure an effect, in this case, NO production from plants, remains to be firmly established in several physiological scenarios. This arises from a series of causes; (1) doubts have arisen over the specificity of widely used 4, 5-Diaminofluorescein diacetate (DAF-2DA)/4-Amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) for NO, (2) no plant nitric oxide synthase (NOS) has been cloned so that the validity of using mammalian NOS inhibitors to demonstrate that NO is being measured is debatable, (3) the NO scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (CPTIO) needs to be used with caution and (4) some discrepancies between assays for *in planta* measurements and another based on sampling NO from the gas phase have been reported. This review will outline some commonly used methods to determine NO, attempt to reconcile differing results obtained by different laboratories and suggest appropriate approaches to unequivocally demonstrate the production of NO.

Nitric Oxide in Plants

Although there have been suggestions of roles for NO in plants for many decades (Fewson and Nicholas, 1960), it was only in the 1990s that a pioneering series of articles by Leshem (1996), Delledonne et al., (1998) and Dürner et al., (1998) clearly established that this was truly a signal in plants. Merely a cursory glance through this special issue will quickly illustrate how NO has emerged as an important signal in plant defence (Leitner et al., 2009; Mur et al., 2006; Wendehenne et al., 2004), stomatal regulation (Neill et al., 2008), root development (Correa-Aragunde et al., 2004) and a range of abiotic stresses (Qiao and Fan, 2008) to derive a far from incomplete list.

However, the most appropriate method to measure NO production, one of the most fundamental aspects of scientific research, is still under controversy. Definitive NO measurements are required to actually establish that it is being produced within a given biological context. Moving on from this, the kinetics of NO production must be determined to set its generation within the context of physiological/cytological/genetic events and the presence of other signals. Further, appropriate treatments with either NO gas or NO donors can be used in large scale experiments such as transcriptomic experiments (Huang et al., 2002) or proteomic based identification of S-nitrosylated or nitrated proteins (Lindermayr et al., 2005; Romero-Puertas et al., 2007).

Problems have arisen for a number of reasons, mostly from the physical properties of NO itself. In the presence of oxygen it has a half-life of 29 sec and can be rapidly scavenged by haem containing proteins, and thiols such as glutathione (Wink et al., 1996). These factors

make NO a very transitory signal. Furthermore, NO effects are concentration dependent (Beligni and Lamattina, 1999; Wink and Mitchell, 1998) which demand that NO must be measured over a broad range of concentrations (pM to mM) to determine its action. Rates of NO production vary enormously with measurements of 0.1 to ~ 200 nmol/h/gram fwt being reported (Mur et al., 2006; Planchet et al., 2005; Rockel et al., 2002). NO production may also be restricted to very few cells, in for example, guard cells (Bright et al., 2006). Thus, measurement methods must be very sensitive to be able to detect NO production from plants. In addition, significant doubts have been expressed as to the specificity of the detection methods, for example the use of DAF dyes (Planchet et al., 2006) which are used by large numbers of NO researchers. This review will briefly describe some of the many available methods to detect NO and consider their advantages and disadvantages. In doing so, we will not attempt to provide an encyclopaedic description of the many methods through which NO may be measured but concentrate on those which have been used by plant scientists. Finally, we will suggest some common approaches that could be followed to yield robust measurement of NO production.

In planta *assays for NO*

Many assays focus on determining NO content within plant tissues to assess the actual concentration that impacts on cellular processes and physiology.

- **The Oxyhaemoglobin Assay**

Early papers on plant NO production utilised a haemoglobin based assay to measure NO production (Clarke et al., 2000; Delledonne et al., 1998). This is a spectroscopic method based on the reaction of oxyhaemoglobin (HbO2) with NO to produce methaemoglobin (MetHb) and nitrate (NO_3^- (Haussmann and Werringloer, 1985). This reaction results in a shift of absorbance from 415–421 nm (HbO2) to 401 nm (MetHb). This is a robust and sensitive assay with a predicted detection limit of 1.3–2.8 nM (Murphy and Noack, 1994).

However, recently this technique has fallen out of favour mostly likely for a series of reasons. Firstly, the production of fresh HbO2 is technically demanding, as it requires haemoglobin oxygenation followed by isolation using chromatography. More seriously, reactive oxygen species can also oxidise HbO_2 to give false readings from the assay. Delledonne et al., (1998) applied catalase and superoxide dismutase to their assays to suppress ROS production but, although possessing an extraordinary high catalytic activity (k_{cat} s^{-1} 40,000,000) the low affinity (Km 25mM) of catalase for its substrate means that the presence of H_2O_2 could remain a confounding factor. This is particularly problematic since the production of NO occur simultaneously, or near simultaneously, with that of H_2O_2 during plant defence responses. Additionally, changes in pH – also a feature of plant defence responses – can affect the assay as can the presence of competing haem containing proteins.

- **The Griess Reaction**

The Griess reaction is one of the most widely used assays for NO detection and represents the basic reaction of relatively cheap commercial kits for NO measurements. The technique was pioneered by Johann Peter Griess (1829–1888), a German organic chemist, who was one

of the founders of the azo and diazo dye industry. Griess suggested that nitrites could be detected by reacting with sulphanilic acid and α-naphthylamine under acidic conditions to yield an azo dye. This remains the basic reaction except that today sulphaniliamide and *N*-(1-naphthyl) ethylenediamine (NED) are used to react with NO_2. The resulting stable water-soluble azodye may be quantified by measuring spectrometric absorption at 520nm. NO can be readily oxidized to NO_2 (usually by CrO_3) so that the basic Griess reaction is used as an indirect assay for NO (Fig. 1). NO_2^- can be further oxidized to NO_3^- which does not form the azodye but the kinetics of NO_2 oxidation are relatively slow and are therefore considered to be insignificant (Ivanov, 2004).

The popularly of the Griess reaction for determining NO in clinical and animal research (Brandonisio et al., 2001; Coulter et al., 2010; Ghafourifar et al., 2008; Tsikas, 2007) has not been mirrored plant research. The Griess assay has been used to determine nitrite ions in cucumber, tomato and wine (Shirinova et al., 1993a; Shirinova et al., 1993b) and perhaps most importantly by Vitecek and co-workers who used the Griess reaction to measure NO production from tobacco (*Nicotiana tabacum*) cultures inoculated with the cell death elicitor, cryptogein, and Arabidopsis mutants exhibiting either increased or decreased NO synthesis (Vitecek et al., 2008). The Vitecek et al., study clearly demonstrated the potential of the Griess reagent so that the relative lack of interest from plant scientists is worthy of brief comment. It may be that its reported lack of sensitivity at 0.5 µM NO (Hetrick and Schoenfisch, 2009; Tracey, 1992) may be deterring its use. However, through a novel implementation of the Griess reaction developed by Vitecek et al., (2008; see below) sensitivities in the nM range were reported. It seems much more likely therefore that the attractiveness of the use of DAF dyes requiring only the use of fluorescent (ideally, confocal)

microscopes has distracted plant researchers from the usefulness of the Griess reagent assay.

- **Diaminofluoresceins (DAFs) fluorescent dyes**

DAF dyes have been very widely used by plant NO scientists (including ourselves Fig. 2) to reveal likely sites of NO generation (Foissner et al., 2000; Krause and Durner, 2004; Lamotte et al., 2004; Prats et al., 2008; Prats et al., 2005). DAF dyes can be readily obtained from commercial sources at a reasonable price and NO can be visualised via fluorescence microscopy. Superficially, it also appears easy to prove that NO is being generated; simply co-apply DAF with either NO scavengers (for example, cPTIO; NO+ cPTIO $\rightarrow$ NO_2 + cPTI) or inhibitors of mammalian NOS.

DAF dyes were first described by Kojima et al., (1998a; 1998b) where they were shown to react with N_2O_3 a by-product of NO oxidation, with a resulting dramatic increase in fluorescence. This was initially commercialised in a diacetate- form (DAF-2DA) which allowed ready uptake by living cells. The diacetate group is removed by cellular esterases leaving the membrane impermeable DAF-2 form available for nitration by N_2O_3 to generate the highly fluorescent triazole (DAF-2T; Fig. 2A) (Kojima et al. 1998b). Encouragingly, no DAF-2T fluorescence was observed with NO_2^-, NO_3^-, H_2O_2 and peroxynitrite ($ONOO^-$) and very low detection limits at 5nM were reported (Kojima et al. 1998b). This dye may be used in flow cytometry (Strijdom et al., 2004) but have mostly been used to image patterns of cellular NO production by fluorescence microscopy.

However, very soon after their development the specificity of DAF dyes have been challenged from various quarters. It may be predicted that the antioxidant ascorbic acid should reduce levels of N_2O_3 and therefore the DAF-2T signal but actually, DAF2 reacts with dehydroascorbic acid (DHA) and ascorbic acid (AA) to generate new compounds that have fluorescence emission profiles similar to that of DAF-2T (Zhang et al., 2002). This problem can only be partially solved through the use of ascorbate oxidase where AA is reduced to DHA and water (Kim et al., 2006) but is also impractical when attempting to measure *in planta* NO where cellular penetration of the enzyme can be expected to be negligible. However, it may be that concentrations of > 5mM AA are required to elicit a detectable fluorescence signal (Planchet and Kaiser, 2006). Planchet and Kaiser (2006) have also noted fluorescence under anoxic conditions which should not be possible given the dependence on the oxidation of NO to N_2O_3; and although, this could not be suppressed with cPTIO it appeared to be dependent on nitrate reductase activity. Other problems, include the differential loading of DAF dyes into different tissues and association with non-NO producing dead cells (Vitecek et al., 2008).

DAF-FM (4-amino-5-methylamino- 2′, 7′-difluorescein) (the diacetate represents the cell permeable version) has been developed as an improved NO sensor to DAF-2DA. The cytoplasmic version of DAF-FM is more photostable than DAF-2 (respectively, ~ 5 nM and ~ 3 nM (Murad, 1999). It has also been suggested that the fluorescent signal of DAF-FM is not affected by pH above 5. However, when measuring the concentration of dissolved NO in water using DAF-FM, Vitecek et al., (2008) noted that the fluorescence obtained with 300nM NO was quenched with increasing pH so that the signals at pH 9 were around half those obtained at pH 5.5. The plant cell cytosol pH is usually around 7.5, with the apoplast and

vacuole being in the region of pH 5.5 but intracellular pH can change dramatically during cellular processes such as the pathogen-elicited HR, root tip growth, nodulation, gibberellic acid and abscisic acid signalling (Kader and Lindberg, 2010; Rengel, 2001; Roos, 2001).

Many of the researchers that used DAF dyes confirmed NO detection with cPTIO that scavenge NO and consequently suppress DAF fluorescence. However, this three way interaction between NO, cPTIO and DAF-2 is not straight forward as its outcome depends on the relative concentration of all three reactants. Carboxy-PTIO is a stable organic radical that was developed by Akaike and Maeda, (1996) and oxidizes the NO molecule to form the $^{\cdot}NO_2$ radical (NO + cPTIO → NO_2 + CPTI). $^{\cdot}NO_2$ radical can react with NO to form N_2O_3 (NO_2 + NO → N_2O_3) which in turn can react with DAF-2 to form fluorescent DAF-2T. This could suggest that cPTIO should increase fluorescence; however this ignores the competing direct oxidation of NO ($4NO + O_2 \rightarrow 2N_2O_3$) to form N_2O_3. Thus, at higher cPTIO concentrations NO will be rapidly converted to $^{\cdot}NO_2$ thereby slowing the formation of N_2O_3. This third order dependence explains the failure of Vitecek et al., (2008) to suppress the fluorescence of DAF-FM in the presence of 380 nM NO with 100 μM CPTIO. However, worryingly, when DAF-FM was allowed to react with NO to form DAF-FM-T (i.e. relatively little free NO), cPTIO proved to be effective masking fluorescence; a feature also observed by Arita et al., (2006). Until the exact nature of this reaction is understood, ideally, cPTIO should not be the only controls used by researchers (see below).

- ***Electron spin resonance***

Electron spin resonance ([ESR], also known as Electron Paramagnetic Resonance [EPR]) is based on observing unpaired electrons in magnetic fields which in the microwave region exhibit a "resonance" between parallel and antiparallel electron spin orientations (Kleschyov et al., 2007). EPR instruments will scan the magnetic field strength until resonance between the parallel and antiparallel states is reached at a given microwave frequency (which will be specific to a given radical) until a signal is observed. As EPR only detects free radical species, it is highly selective to NO over all other products of N oxidation (Kleschyov et al., 2007). However, the highly ephemeral nature of the NO radical entails using specific "spin-trap (ST)" chemicals which give longevity to a (in this case) NO –dependent radical signal ($NO^{\cdot}$ + ST → $NO\text{-}ST^{\cdot}$). The detection limits of EPR are in the order of pmol (Weaver et al., 2005).

Iron-dithiocarbamates have been often used for ESR which exploits the high affinity of NO for iron (Van Doorslaer and Desmet, 2008). Iron-dithiocarbamates ST ($Fe(S_2CN\text{-}R\ R')_2$) ,exist with a range of side groups (R and R' can be either be methyl-, ethyl-, glucamine-, sarcosine- or amino acids (Weaver et al., 2005). The different properties conferred by these side groups are useful for targeting to for example, hydrophobic membranes in the case of Fe-diethyldithiocarbamate or extracellular fluids with the polar Fe-*N*-methyl-d-glucamine dithiocarbamate (Kleschyov et al., 2007).

EPR has been used in plants to report NO production from pollen (Bright et al., 2009), sorghum embryonic axes (Jasid et al., 2008) and also Arabidopsis infected with bacterial pathogens (Modolo et al., 2005). Further, lipophillic ST have been used to show NO effects on the oxygen-evolving complex of photosystem II from cyanobacterium *Synechococcus elongates* (Sarrou et al., 2003). In a particular, interesting paper, Cao et al., (2005)

demonstrated the detection of NO and reactive oxygen species following the co-application of different ST. Such successes notwithstanding, EPR has not been widely used by plant scientists due to the inherent costs of EPR resonators and the considerable expertise required in order to exploit this platform; such that most biological studies involve collaborations with physics departments. Experimentally, EPR whilst excellent for one-off readings is difficult to apply to continuous, long term, reading of the same plant sample (Xu et al., 2005).

- **NO electrodes**

NO electrodes have been widely used by clinical scientists as they represent a relatively cheap and easy means to detect NO (Davies and Zhang, 2008). The "classical" NO electrode consist of a platinum Teflon coated working electrode and a Ag/AgCl reference electrode, both encased in a glass micropipette filled with 30 mM NaCl/0.3 nM HCl solution except for an open end covered with and NO- permeable membrane. These can be made from different compounds such as chloroprene rubber, cellulose acetate, collodion/polystyrene, PTFE, and phenylenediamine (Davies and Zhang, 2008). Upon passage of an electric current NO is detected based on its oxidation at +0.8 to +0.9 V compared to the reference electrode (Shibuki, 1990). Reported sensitivities of NO electrodes have been in the order of 10^{-20} mol of NO in single cells (Malinski and Taha, 1992).

Leshem, (1996) demonstrated that an NO electrode could be used in plants to detect NO simply pushing the electrode into fruit. However, several plant organs are not amendable to such intervention, thus, electrodes have been most often used in plant tissue culture.

Electrodes have been used to reveal NO production during cadmium induced cell death in tobacco BY-2 cells (Ma et al., 2010) and cultures of green alga *Chlamydomonas reinhardtii* (Sakihama et al., 2002).

In a very interesting *in planta* study NO microelectrodes were inserted into pelargonium leaves and this allowed the detection of the rapid generation of NO within minutes of wounding followed a second wave at 2h. Judicious positioning of the microelectrodes revealed that NO generation was restricted to the site of injury (Arasimowicz et al., 2009). Clearly, this study shows the possible wider utility of NO electrodes in plant science.

- Mass Spectroscopy

Another method surprisingly neglected by plant scientists was described by Conrath et al. (2004). This Mass Spectrometric approach allowed the on-line detection of NO from either tissue cultures or whole plants. In restriction inlet mass spectrometry (MIMS) configuration, a membrane separates the mass spectrometer (MS) from the tissue culture but allow the diffusion of small molecular weight gases such as NO. MIMS was used to detect NO production using a membrane inlet to allow free diffusion of NO from tissue cultures of either tobacco or soybean inoculated with HR-eliciting or disease forming strains of *Pseudomonas syringae*. In a restriction capillary inlet MS (RIMS) configuration NO was sampled in the gaseous phase from cuvettes sprayed with 20m M $NaNO_3$. A particularly attractive of RIMS/MIMS is that they are able to distinguish between different N isotopes so that on supplementation of (for example) cultures with likely substrates for NO generating enzymes (for example N^{15} – labelled nitrate/nitrites/polyamines/hydroxylamines) their

contribution (if any) to the NO produced can be estimated. Given the prevalence of MS infrastructure in many Institutes and Universities, there should be many opportunities for plant NO scientists to exploit the RIMS/MIMS approaches.

Ex planta *assays for NO : Detection of gaseous NO*.

Although NO is readily soluble in water (7.4 mL/100 mL), it easily volatilises into the gaseous phase (critical temperature: -93 ^{o}C; critical pressure: 64.85 bar). Thus, a range of approaches have emerged to measure gaseous NO concentrations which are attractive as they can provide on-line, *in planta* measurements of the kinetics of NO production. However, these approaches should, of necessity, be seen as only an indicator of *in planta* NO production as sampling from the atmosphere represents "lost" NO - in terms of plant signalling. In all of the approaches described below it should also be noted that the possibility of artifactual readings arising from other volatiles – in many cases water vapour - needs to be considered.

- **Chemiluminscence**

By far the most well-established approach to measure gaseous NO is the chemiluminescent assay (Fig. 3) which is based on its reaction with O_3 to yield light photons. This is a two stage reaction whereby the reaction of NO with O_3 produces excited-state nitrogen dioxide (NO_2^*), which emits a photon upon relaxation to the ground state: The emitted light, at > 600 nm wavelength is measured with a photomultiplier tube (PMT) with an intensity that is proportional to the amount of NO (Fig. 3). The results are highly specific for NO as, although chemiluminscence can result from the reaction of O_3 with ethylene and sulphur compounds,

these reactions emitted at 440-470 and < 400 nm respectively, much lower than the specific NO/O_3 reaction. The chemiluminscence approach exhibits excellent sensitivity with limits of detection as low as 20-50 pmol (Byrnes et al., 1996) and need only minimal equipment which has contributed to its commercialisation as robust platform for NO measurement. Within plant science, the chemiluminscent platform has been mostly utilised by the Kaiser group (Wurzburg, Germany) to provide significant insights into NO biology. Thus, production of NO during anoxia (Rockel et al., 2002) synthesis of NO from hydroxylamines (Rumer et al., 2009) and the NADPH-dependent reduction of nitrite to NO in mitochondria via a non-NR mechanism (Gupta et al., 2005) have been demonstrated.

Laser based infrared spectroscopy

NO may also be measured using techniques that are based on infrared (IR) absorption. These approaches make use of the specific absorption of NO at 5.3 µm (1876 cm^{-1}) (Rothman et al., 2005). Two platforms will be discussed here – laser photoacoustic detection (LAPD) and Quantum Cascade Lasers (QCL) based system.

LPAD is based on the detection of evolved gases as they adsorb rapidly chopped infrared light. The resulting absorption-relaxation results in pressure variations to generate sound which is detected by a microphone, located within the photoacoustic chamber. The photoacoustic cell is placed inside the cavity of the laser to achieve a high effective laser power. The photoacoustic signal (“sound”) generated in the cell depends on the property of the gas, i.e. the absorption coefficient and is proportional to the concentration of absorbing trace gas molecules (Fig. 4A) (Cristescu et al., 2008).

NO detection by LAPD involves the use of a CO laser whose emission covers the spectral region from 4.6 to 8.2 mm. Following up pioneering work by Leshem and Pinchasov,(2000), our use of LAPD involved measuring the photoacoustic signal over five laser lines (wavenumbers) to remove any possible contribution to the NO signal by water, ethylene and NO_2 (Mur et al., 2005). LAPD allowed us to measure NO production from tobacco and Arabidopsis plants challenged by bacterial pathogens (Mur et al., 2006; Mur et al., 2005). With a delay of only 2.5 min between *in planta* emission and detection within the photoacoustic chambers, the measurements were near-contemporaneous and continuous. We also demonstrated a detection limit of around 20 pmol which make LAPD comparable to the chemiluminescence's NO detection platform (Mur et al., 2005).

Although this represented a significant advance in plant NO measurements there are considerable logistical problems associated with LAPD (Fig. 4B). The requirement for physically large, specialised equipment hardly makes the LAPD platform one that could be widely employed by many groups – unless the experiments were sufficiently portable to allow measurements to be made at a single place, for example the Trace Gas Facility at Radboud University (Nijmegen, The Netherlands). Further, although LAPD is a sensitive technique it is unlikely to detect NO production from a smaller numbers of cells, for example, stomata; and naturally; given that NO is being detected in the gas phase, no spatial information can be deduced. Technical limitations centre around the need to ensure the removal of water vapour whose photoacoustic signal will interfere with the detection of a range of trace gases. In the Mur et al., (2005), water was removed by a cold trap but it may be possible to use a calcium hydrate filter.

Quantum cascade lasers

Nijmegen guys; I cannot write this section without making a mess. Could you help?

The use of QCL spectroscopy is a relatively new to plant science….? (Fig. 5).

The QCL-based spectrometer is equipped with an astigmatic multi-pass (76m) absorption cell for wavelength modulation spectroscopy on NO.

It is cooled with a Peltier element to a temperature of −30 ◦C.

In order to test the performance of QCL, we compared it to the chemiluminescent NO detection platform (Fig. 6). To this aim, the tomato ABA mutant *sitiens* was spray-inoculated with a 10^5 conidia/mL suspension of *Botrytis cinerea* to run-off. The plants were allowed to air dry for 1 h after which they were placed within a 2 litre volume cuvette. The chemiluminescent platform required an input flow rate of the carrier air of at least 12 L/h whilst the QCL is limited to around 1 L/h. Thus, we passed 14 L/h through the cuvette which

was subsequently split into flows of 13 L/h to the chemiluminescent platform and via a Mass Flow Controller (MFC) to 1 L/h to the QCL (Fig. 6A). It is important to note that irrespective of the split flows both platforms are measuring NO content from the same cuvette which are normalised to rates of production per litre. Our detection of identical NO levels using QCL and chemiluminescent system in this experiment, demonstrated the validity of the latter (Fig. 6B). Following this research we are currently preparing a detailed description of NO detection in *B. cinerea* infected tomato (*Solanum esculentum*) using QCL (Sivakumaran et al., in prep).

In considering measurements of gaseous NO from plants, we must take into account instances where there have been mismatches between reported NO production as detected using DAF- dyes and the chemiluminescent approach (Planchet and Kaiser, 2006; Planchet et al., 2006). Thus, there is an apparent lack of detection of NO in cryptogein treated plants and cultures using chemiluminescent detection compared to the use of DAF dyes and NO electrodes (Planchet et al., 2006). The difficulties of using DAF dyes have been outlined above but before using such considerations to dismiss data derived from these dyes it is worth considering the preponderance of data supporting the NO is generated. It should not be denied that oxyhaemoglobin assays and DAF dyes can indeed measure NO, which is supported by the fact that large numbers of important studies each using different methods have noted NO generation during the HR (Clarke et al., 2000; Conrath et al., 2004; Delledonne et al., 1998; Foissner et al., 2000; Mur et al., 2005) and also cryptogein treatment (Foissner et al., 2000; Vitecek et al., 2008). Most compellingly, strongly, we should consider independent indicators of NO generation during the HR. Recently, many groups have been focusing on protein S-nitrosylation and nitration during the plant defence against

pathogens (Romero-Puertas et al., 2007; Tada et al., 2008), which, besides yielding some fascinating observations, represents an independent validation that NO is produced. Additionally an important point of the Vitecek et al., (2008) study was that their detection of NO production from the gas phase of cryptogein inoculated tobacco suspension cultures was based on flow rates of 2.4 L/h and also included a substantial "signal integrative" step as the azodye accumulated in the second trap (Fig 1B). The reader should also note that in our comparison between QCL and chemiluminescent platforms (Fig. 6A), the flow of 14 L/h represents are considerable dilution of the signal compared to our usual 1-1.5 L/h (Mur et al., 2005). Thus, when using our usual flow rate we measured NO concentrations in the region of 800 ppbv (data not shown). This far exceeds the levels we detect from a bacterially elicited HR in Arabidopsis or tobacco which have never exceeded 80 ppbv (data not shown). Thus, our measurement of HR would have been diluted to below the detection limits if we had used flow rates of 14 L/h. We are not suggesting that this invalidates the otherwise excellent chemiluminescent system but that configurations which use lower flow rates should be used or the integration period over which a signal is collected should be increased.

When comparing QCL and chemiluminescent systems we made a serendipitous observation which has bearing on gaseous NO measurements from the air (Fig. 7). When including the module with soil and Arabidopsis rosette in the cuvette, we detected more NO production when the plant was cut and removed than when it was present. When the excised plant was reapplied to the surface of the soil, NO production was again reduced (Fig. 7A). This suggested that the soil – or more likely the soil microbes - was a major source of NO (5 sec microwave of the soil destroyed all NO generation, data not shown). Whilst this could have reflected a masking of the NO signal by a plant volatile(s) we have since associated this loss

of NO with its oxidation by plant haemoglobins ([Hb] Mur et al. paper submitted). This suggests that when measuring NO care must be exerted to make sure that as much of the plant material under assessment is producing NO; otherwise oxidation by Hb would reduce the gaseous “lost” NO signal. In our case we have been fortunate that we have always used heavily-inoculated tobacco leaves (Mur et al., 2005, 2008), Arabidopsis rosettes (Mur et al., 2006) or tomato seedlings (Fig. 6). We suggest that wherever possible, experimenters seeking to measure NO from the gas phase should maximize the proportion of plant material producing NO.

In passing, these observations have implications regarding the sources of NO generation. Whilst nitrate reductase (NR, Modolo et al., 2005)), NOS-like enzymes (Corpas et al., 2009), polyamine reducing enzymes (Yamasaki and Cohen, 2006), hydroxylamine reducing enzyme (Rumer et al., 2009) and chemical reduction of nitrate (Bethke et al., 2004) are clearly sources of NO, a suppression in Hb expression would increased concentrations of *in planta* NO. We would predict that such could be most relevant in roots (Hb will oxidize NO at very low partial pressures of O_2, (Garrocho-Villegas et al., 2007), or in leaves of low lying rosette types plans such as Arabidopsis. Equally, localised suppression of Hb could aid to further elevate NO concentration when generated at, for example, a HR.

NO measurements – some recommendations.

Our consideration of the preferred methods used by plant scientists to detect NO has highlighted their power to provide some cutting edge insights into NO biology. Equally, it has

revealed some considerable problems with each technique – whether this be doubts as to their specificity, simple logistical costs or the inappropriateness to a system under investigation.

Until other NO reporting fluorescent dye become available, we accept that it is unavoidable that DAF-based dyes will continue to be used. The ability that DAF stains have to report NO generation within discrete cell types is currently unparalleled. Confirmation that NO is indeed being measured should follow the following steps. Firstly, non-DAF treated materials should be imaged to ascertain background fluorescence which should be quantified and the increase on application of DAF should be expressed as a factor of the background (see Prats et al., 2005). Next, attempts should be made to suppress the putative NO fluorescence signal with cPTIO and mammalian NOS inhibitors if desired. Should these reduce the fluorescence signal, this should not be taken as definitive proof (for the reasons mentioned above) but clearly, no suppression would indicate that NO is not being measured. Ultimate confirmation can involve the independent measurement of DAF-2T using high-pressure liquid chromatography (HPLC; Kaiser,W. [Wurzburg] pers. comm.). If well-characterised NO mutants (see Vitecek et al., 2008) or transgenic Hb lines (for example, hmpX over-expressing lines; Boccara et al., 2005)) are available, these should be used in preference to any other control. Alternatively, NO production should be measured using more than one technique. A good example of this approach is provided by Bright et al., (2008) where NO production from rehydrated pollen was measured using DAF dyes and also EPR. This ethos also underpinned the approach of Planchet and Kaiser (2006) who attempted to compare NO signals from cryptogein treated plant tissues using the chemiluminensce approach and DAF-2DA dyes. An illustration of this approach from our own data is given in Fig. 8.

We have reported NO generation from *Blumeria graminis* f. sp. *hordei* (powdery mildew) challenged Barley cultivar P-01 (Prats et al., 2005). In this cultivar *B. graminis* elicits single epidermal cell death or forms cell wall papillae, both of which are associated with NO generation. Such very subtle patterns of NO generations represent a challenge for a gas based NO detection system. However, NO emissions determined using QCL closely matched the patterns previously reported using DAF-2DA (Fig. 8).

Obviously, many groups do not have access to a chemiluminescent NO detector, or such specialised equipment as an EPR resonator or LAPD, QCL devices. In such circumstances, we would urge such groups to consider using NO electrodes (where an *in planta* measuring approach has been demonstrated (Arasimowicz et al., 2009)) or, even more simply, use the relatively neglected (by plant scientists) Griess reagent assay. Vitecek et al., (2008) have demonstrated how this can be used to measure NO from the gaseous phases and allowing accumulation of azodye over time (Fig. 1B). This dramatically increases the sensitivity of the system by increasing the length of time over which the NO signal can be integrated.

In summary, we suggest that adoption of robust NO measurement approaches will assuage much of the controversy that is a feature of much of plant NO research.

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Legends:

Figure 1: Griess reagent method of NO detection

(A) Nitric oxide (NO) is oxidised to nitrite (NO_2^-), by CrO_3. NO_2^- reacts with sulphanilamide to form a diazonium salt intermediate. The diazonium salt is then coupled to *N*-(1-naphthyl) ethylenediamine (NED) to form the stable water-soluble azo dye (λmax 540 nm). **(B)** Schematic of the apparatus used to detect NO in the gaseous phase based on the Griess reaction as developed by Vitecek et al., (2008). A gas flow passes through a humidifier and into the sample chamber. Any NO_2 (or HNO_2) in the airflow is captured in the first trap which contains sulphanilamide and NED but NO progresses into the oxidiser tube where CrO_3 oxidizes the NO to NO_2 which is detected by a second Griess reaction ion trap 2.

Figure 2: Use of DAF dyes to suggest localised generation of NO in powdery mildew (*Blumeria graminis* f.sp. *hordei*) challenged Barley (*Hordeum vulgare* cv Pallas 01 [P-O1]) and *Arabidopsis thaliana* Col-0.

(A) On application 4, 5 diaminofluorescein diacetate (DAF-2DA) is readily taken up into cells where cytoplasmic esterases remove the acetate groups (to generate 4, 5 diaminofluorescein; DAF-2) preventing movement back out of the cell. DAF-2 can react with N_2O_3, an oxidation product of NO to generate the highly fluorescent DAF-2T (triazolofluorescein). **(B)** Background auto fluorescence and **(C)** fluorescence on treatment with DAF-2DA in barley (*Hordeum vulgare* cv Pallas 01 [P-O1] harbouring resistance gene *Mla1*) at sites of attack with powdery mildew (*Blumeria graminis* f. sp. *hordei* race CC1) at 15

h following infection. Arrowed is a cell undergoing a hypersensitive response (HR). All other sites of fluorescence are associated with developing papillae and a stoma. Bar = 50 μm. **(D)** Site of attack in *Arabidopsis thaliana* by *B. graminis* f. sp. *hordei* race CC1 at 15 h following infection. Following application of DAF-2DA fluorescence at the site of papilla formation is arrowed (red). The papilla is occurring on the underside of an appressorial germ tube. The condium (spore) is out of focus but indicated by a dotted oval. Note that the papilla is the focus of vesicle targeting. Fluorescence is also observed in the stomatal guard cells (blue arrow). Bar = 50. DAF-2DA treatment methods and confocal microscopy were as detailed in Prats et al, (2005).

Figure 3: Chemiluminescent detection of nitric oxide

Schematic diagram of a chemiluminescence-based nitric oxide (NO) assay. A carrier gas is passed through a sample cuvette where NO production is occurring and then on to the reaction cell within the NO analyzer (the Sievers Nitric Oxide Analyzer [NOA 280i] analyzer is depicted). In the reaction cell, NO reacts with ozone (O_3) to form excited-state nitrogen dioxide (NO_2*), which emits a photon when relaxing to its ground state (NO_2). The emitted light passes through an optical filter and is amplified in a photomultiplier tube (PMT) and quantified.

Figure 4: NO detection in the gaseous phase using laser photoacoustic detection (LAPD)

(A) Plant material [here shown as a red pepper fruit] is carried on an airflow (in the region of 1-2.5 L/h) by mass flow controllers (not shown) . Water vapour in the gas flow is removed using a Peltier cooling element (-5 ^{0}C) and a cold trap (-80 ^{0}C) (not shown), prior to passage into the photoacoustic cell. The photoacoustic cell was inserted in a laser cavity, to improve laser power and thus detection sensitivity. To generate a photoacoustic signal the laser light was modulated by a chopper (modulation frequency 1000 Hz). In the case of NO absorption and relaxation in response to chopped laser light (1876 cm^{-1}) to generate the photoacoustic signal (S). S is defined as a the factor of the cell constant (F), microphone sensitivity (Sm) and the absorption coefficient (a) of the gas, all of which are constant, as well as laser power (P) which is known. **(B)** Image of a CO laser used for LAPD.

Figure 5: Detection of nitric oxide using a quantum based laser based approach.

Any help here quys….?

Figure 6: Comparison of quantum cascade laser (QCL) and chemiluminescent based nitric oxide detection platforms

(A) A two week old tomato (*Solanum esculentum*) mutant *sitiens* was sprayed to run off with 10^5 condia/mL of *Botrytis cinerea* (strain IMI 169558, Thomma et al., 1997) in potato dextrose broth. After a period of 1 h to allow air drying the infected plants were placed in a 2 L capacity cuvette. Due to the requirements of the Sievers Nitric Oxide Analyzer [NOA 280i] chemiluminescent analyser that was used the flow rate was set to 14 L/h. The airflow was divided to feed into the NOA280i and into QCL NO detector. Due to the constraints of the QCL a mass flow controller (MFC) limited the flow rate to 1 L/h. As both signals are normalised to 1 L/h the signals from QCL and NOA 280i are directly comparable. **(B)** NO production from *B. cinerea* infected tomato plants as measured using QCL and NOA 280i chemiluminescent systems.

Figure 7: Reduced NO detection with the inclusion of non-infected plant tissue

(A) NO production from a 5 week old (short day 8 h light) Arabidopsis Col-0 plant grown on a module of Levingtons M2 compost (approximately $27cm^3$. Pictured; bar = 1 cm) and from the same soil from which the plant has been removed by cutting the stem at the soil surface. The rosette was therefore left intact. Reapplication of the cut rosette to the soil surface reduced NO production. **(B)** NO production from tobacco (*Nicotiana tabacum* cv. Samsun NN) leaves inoculated with (1 x 10^6 bacteria/mL 10 mM $MgCl_2$) *Pseudomonas syringae* pv. *phaseolicola* strain 1448A either over the entire leaf (100%) or, ~75%, ~ 50%, ~25% of the leaf at 6 h after challenge. Results are given as mean (n= 3 +/- SE) nmol/h/g fwt. Pictured are leaves either entirely infected (100 %), or, ~75%, ~ 50%, ~25% inoculated with *P. s.* pv. *phaseolicola* at 24 h after challenge so that tissue collapse illustrates the extent of

inoculation (Bar = 1cm). Based on the mean levels of NO production with 100% leaf inoculation it is possible to predict the "theoretical" production levels with leaves which have been inoculated over lower % of their area. Note that actual NO production rates are considerably lower than the theoretical. Our data suggests that this is due to oxidation of NO produced by infected tissue by the surrounding uninfected tissue.

Figure 8: Nitric oxide detection in mildew based barley using DAF-2DA dye and quantum cascade laser (QCL)

(A) NO production over a 43 h period in 1 week old barley (*Hordeum vulgare* cv. Pallas 01 [P01]) uninfected and infected seedlings. The light-dark periods are indicated. The infection protocol was as described in Prats et al. (2005). Note, the increases in NO in uninoculated controls (labelled "a" and "c" on Fig. 8A) which correlated with the periods where stomatal closure was being initiated (data not shown). The increased NO production at points a and c were significantly (P <0.01) different to NO production in the middle of the light period (labelled "b" on Fig. 8A). **(B)** Autofluorescence and fluorescence after DAF-2DA treatment of the same samples taken at 12 h after infection (hpi), i.e. during papillae formation and at 14 hpi when hypersensitive response (HR) is being initiated and after formation of the HR. Note that these events are occurring mostly in the epidermal cell layer. Comparing (A) and (B), note that the increases in NO production as detected using QCL correlated with increases in DAF-2DA associated fluorescence but not autofluorescence. Increases in fluorescence with

DAF-2DA but not autofluorescence could be suppressed upon treatment with 250 μM cPTIO (see Prats et al., 2005).

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